


## METHODOLOGY

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# Utilising the native plasmid, pCA2.4, from the cyanobacterium *Synechocystis* sp. strain PCC6803 as a cloning site for enhanced product production

Patricia Armshaw<sup>1\*</sup> , Dawn Carey<sup>1</sup>, Con Sheahan<sup>2</sup> and J. Tony Pembroke<sup>1</sup>

## Abstract

**Background:** The use of photosynthetic autotrophs and in particular the model organism *Synechocystis* PCC6803 is receiving much attention for the production of sustainable biofuels and other economically useful products through metabolic engineering. Optimisation of metabolic-engineered organisms for high-level sustained production of product is a key element in the manipulation of this organism. A limitation to the utilisation of metabolically-engineered *Synechocystis* PCC6803 is the availability of strong controllable promoters and stable gene dosage methods for maximising gene expression and subsequent product formation following genetic manipulation.

**Results:** A native *Synechocystis* PCC6803 small plasmid, pCA2.4, is consistently maintained at a copy level of up to 7 times that of the polyploid chromosome. As this plasmid is stable during cell division, it is potentially an ideal candidate for maximising gene dosage levels within the organism. Here, we describe the construction of a novel expression vector generated from the native plasmid, pCA2.4. To investigate the feasibility of this new expression system, a yellow fluorescent protein (YFP) encoding gene was cloned downstream of the strong P<sub>trc</sub> promoter and integrated into a predicted neutral site within the pCA2.4 plasmid. The stability of the integrated construct was monitored over time compared to a control strain containing an identical YFP-expressing construct integrated at a known neutral site in a chromosomal location.

**Conclusions:** A significantly higher fluorescence level of the yellow fluorescent protein was observed when its encoded gene was integrated into the pCA2.4 native plasmid when compared to the isogenic chromosomally integrated control strain. On average, a minimum of 20-fold higher fluorescence level could be achieved from integration into the native plasmid. Fluorescence was also monitored as a function of culture time and demonstrated to be stable over multiple sub-cultures even after the removal of selective pressure. Therefore, the native small plasmid, pCA2.4 may be utilised to stably increase gene expression levels in *Synechocystis* PCC6803. With the complementary utilisation of an inducible promoter system, rapid generation of commodity-producing *Synechocystis* PCC6803 strains having high level, controlled expression may be more achievable.

**Keywords:** Yellow fluorescent protein (YFP), pCA2.4, Gene dosage, Gene expression, *Synechocystis* PCC6803

\*Correspondence: [patricia.armshaw@ul.ie](mailto:patricia.armshaw@ul.ie)

<sup>1</sup> Molecular and Structural Biochemistry Laboratory, Department of Chemical and Environmental Sciences; Materials and Surface Science Institute, University of Limerick, Limerick, Ireland  
Full list of author information is available at the end of the article

## Background

Reserves of fossil fuels are declining while greenhouse gas emissions are on the rise [1]. This has led to an increasing emphasis on the research of biofuel production systems that harness CO<sub>2</sub>. For this reason, interest in the utilisation of cyanobacteria for biotechnological applications has been increasing [2]. Cyanobacteria have the capacity to convert carbon dioxide to organic carbon metabolites and through genetic modification of these organisms; these metabolites can be converted into commodities for biofuel applications [3, 4].

Currently, industrial utilisation of such modified organisms to produce biofuels and other commodities are uneconomic due to the high cost of production and low titers often initially produced [3]. Consequently, efforts to utilise cyanobacteria economically require significant increases in production rates of the target product [4]. *Synechocystis* sp. strain PCC6803, henceforth referred to as *Synechocystis* PCC6803, is commonly used as a model cyanobacterium due to its relative ease of genetic manipulation; its ability to grow both autotrophically and heterotrophically with minimal light; and ability to undergo natural transformation [5]. In addition, this strain can express several well-characterised *Escherichia coli* promoters, although with altered control in many cases [6]. Tightly regulated *E. coli* promoters can be leakier and of lower strength in *Synechocystis* PCC6803 [7]. In addition, *Synechocystis* PCC6803 is polyploid and the slow growth rate of the strain can significantly lengthen the time required for any genetic modification [8]. In spite of these recognised difficulties, *Synechocystis* PCC6803 has been shown to have the potential to produce many economically interesting products. For a detailed review, please see Dexter et al. 2015 [3] and Angermayr et al. 2015 [4]. However, the issue of initial low production levels of target compounds of interest is a limiting concern [9].

A general method for increasing the production of target compounds is to alter the expression levels of the specific genes required for product formation to increase the carbon flux directed towards the commodity production [10]. Indeed, if the expression level of the necessary enzymes is not in excess, other methods such as increasing the carbon flux coming into the cell will likely not lead to increased commodity titers and the increased carbon flux is likely to be directed towards biomass formation [11]. Studies have found that utilising multiple copies of the necessary genes for production within the chromosome or known strong promoters can increase product titers [10, 12]. However, the generation of modified strains with multiple chromosomal insertions can have several drawbacks. With the slow growth rate of *Synechocystis* PCC6803, each separate modification can be time consuming to generate with multiple modifications

requiring many rounds of cloning, transformation, segregation and selection especially if marker-less clones are required. Several different antibiotic cassettes can be required to build the constructs which can be of concern in pilot scale photobioreactors due to the cost and controversy concerning their use [13]. In addition, multiple different neutral sites are needed to provide multiple insertions. All this can be significantly time-consuming and multiple-cassette containing strains may be unstable and difficult to maintain, losing copies of the inserted cassettes over time [14]. Ideally, integration of a single cassette into a high-copy number plasmid that would be stably maintained within the cell may allow maximisation of gene expression while minimising the time required for strain generation. Utilisation of a native high-copy number plasmid that is naturally and consistently maintained could potentially be more stable than a high-copy number non-native plasmid that has been transformed into the strain.

pCA2.4 is a small cryptic native plasmid found to be consistently present in *Synechocystis* PCC6803 [15]. It is stably maintained within the cell indicating that the plasmid may provide some unknown essential function. It is 2378 bp in size and contains two predicted ORFs with little known about its function. One of the two ORFs, *repA*, has an annotated function that suggests the plasmid replicates by a rolling circle mechanism [15]. The copy number of this plasmid when analysed [16] was found to change significantly depending on growth phase and nutrient conditions utilised. Under autotrophic conditions, the plasmid copy number per chromosome during exponential phase was approximately 0.75 changing to 5.41 during stationary phase. Utilisation of mixotrophic conditions (5 mM glucose) led to higher copy numbers during both phases of growth: 6.26 (exponential) and 7.39 (stationary) [16].

As pCA2.4 is stably maintained within *Synechocystis* PCC6803 without any recognisable selective pressure, integration of a production cassette within the plasmid, if stable, would have the potential to increase the gene copy number up to 7 times higher than that of a chromosomal integration site. In addition, the expression level of the plasmid could be controlled by modification of the growth conditions or by utilisation of promoters of varying strength. Modified strains could be more easily generated by carrying out transformation and strain segregation under low plasmid copy number conditions. Maximising expression could be achieved by maintaining the cell culture at stationary phase, under mixotrophic conditions if desired or by the utilisation of an inducible promoter system such as a riboswitch [17]. Several P<sub>trc</sub> riboswitches have recently been characterised in cyanobacteria and tightly regulated expression shown to be

possible [18]. However, the maximal expression levels of the P<sub>trc</sub> promoter with a riboswitch are reduced indicating that gene dosage with a riboswitch system would be necessary for reasonable production rates to be achieved [18].

To investigate the possibility of utilising this native plasmid as a stable neutral site for maximising gene expression within *Synechocystis* PCC6803, several constructs, including one that contained a yellow fluorescent protein encoding gene under the control of a constitutive P<sub>trc</sub> promoter (P<sub>trc</sub>), were generated (Table 1). These constructs were integrated at both a chromosomal (*psbA2*) and a putative pCA2.4 neutral site location. The relative fluorescence levels were extensively examined to determine if pCA2.4 could be utilised to express high levels of desired genes under long-term culturing conditions.

## Results and discussion

### Selection of a suitable reporter gene and promoter construct

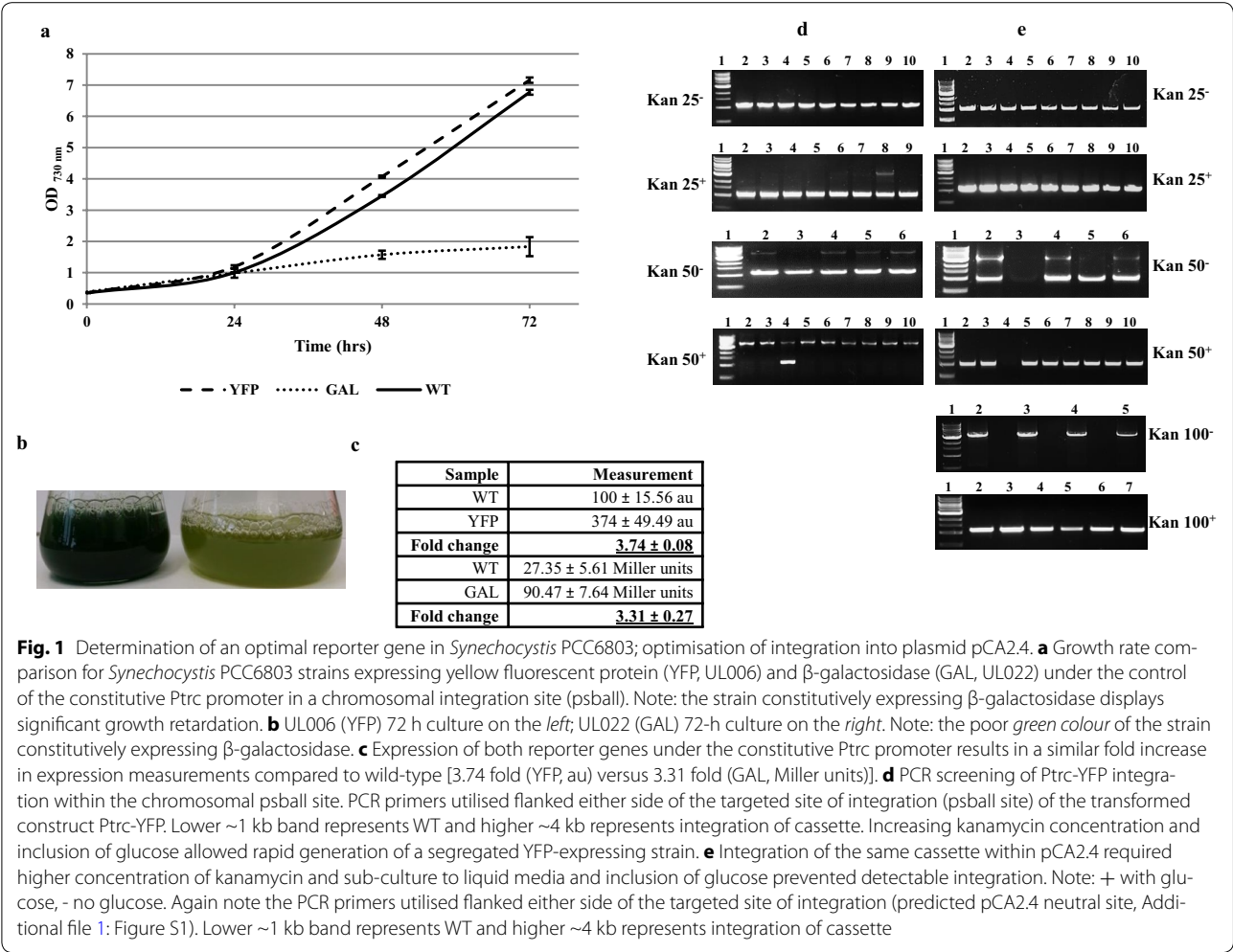
The objective of this work was to determine if pCA2.4 could be utilised for stable high-level expression of genes of interest in *Synechocystis* PCC6803. To determine the true maximal gene expression level that can be reached with integration into pCA2.4 without toxicity effects, two

reporter genes were considered (Fig. 1). While both yellow fluorescent protein [YFP] and  $\beta$ -galactosidase (GAL) expression were detectable at similar levels with a chromosomal integration (Fig. 1c), it was found that integration of the  $\beta$ -galactosidase gene within the chromosomal neutral site resulted in significant growth retardation (Fig. 1a). While  $\beta$ -galactosidase has previously been utilised in *Synechocystis* PCC6803 as a reporter gene [19, 20], in this case it was expressed under the control of a very strong constitutive promoter, P<sub>trc</sub> and as such the expression of  $\beta$ -galactosidase under the control of this promoter was found to be adverse. Integration of a  $\beta$ -galactosidase expressing construct into pCA2.4 could lead to even higher expression levels, hence it was determined that YFP, which displayed a similar growth rate to wild-type when expressed under the control of the P<sub>trc</sub> promoter within the chromosomal neutral site would be a more favourable reporter gene to be utilised throughout this analysis.

Similarly, an initial screening with both  $\beta$ -galactosidase and YFP expressed under the control of different promoters was carried out with the aim of determining a promoter-gene construct that would give a strong detectable signal when integrated in the chromosomal control neutral site—*psbA2* (Fig. 2). Three promoter-gene

**Table 1 Bacterial strains, genetic elements, plasmids and primers used in this study**

Strain	Genotype/phenotype	Source
TOP10	<i>E. coli</i> strain. F <sup>-</sup> , <i>mcrA0</i> , $\Delta$ ( <i>mrr-hsdRMS-mcrBC</i> ), $\phi$ 80 <i>dlacZ58</i> (M15), $\Delta$ <i>lacX74</i> , <i>recA1</i> , <i>araD139</i> , $\Delta$ ( <i>araA-leu</i> )7697, <i>galU</i> <sup>-</sup> , <i>galK0</i> , <i>rpsL</i> <sup>-</sup> (Str <sup>R</sup> ), <i>endA1</i> , <i>nupG</i> <sup>-</sup>	Bio-Sciences, Dun Laoghaire, Dublin, Ireland
AA314	Wild-type <i>Synechocystis</i> PCC6803 strain	K. Hellingwerf, UvA, Amsterdam, The Netherlands
UL006	PCC6803 transconjugant with P <sub>trc</sub> -YFP, <i>psbA2</i> locus, Kan <sup>R</sup>	This study
UL007	PCC6803 transconjugant with P <sub>trc</sub> -YFP-E*, <i>psbA2</i> locus, Kan <sup>R</sup>	This study
UL008	PCC6803 transconjugant with P <sub>psbA2</sub> -YFP, <i>psbA2</i> locus, Kan <sup>R</sup>	This study
UL018	PCC6803 transconjugant with pCA2.4-P <sub>trc</sub> -YFP, Kan <sup>R</sup>	This study
UL022	PCC6803 transconjugant with P <sub>trc</sub> -GAL, <i>psbA2</i> locus, Kan <sup>R</sup>	This study
UL023	PCC6803 transconjugant with P <sub>trc</sub> -GAL-E*, <i>psbA2</i> locus, Kan <sup>R</sup>	This study
Plasmid/ICE	Genotype/phenotype	Source
pEYFP	YFP source plasmid	Clontech, CA 94043, USA
pSF-OXB20-BetaGal	$\beta$ -galactosidase gene source plasmid	Oxford Genetics Ltd, Oxfordshire, OX25 5HD, UK
ICE R391	Kanamycin resistance gene source Integrative conjugative element	[26]
pUC18	Am <sup>R</sup> backbone plasmid	Sigma-Aldrich, Arklow, Wicklow, Ireland
pCR2.1-pTRC-riboswitchE*	pTRC-E* and pTRC source plasmid	Eurofins mwg/operon, 85560 Ebersberg, Germany
P <sub>psbA2</sub> -YFP	P <sub>psbA2</sub> promoter, YFP, <i>psbA2</i> integration site, Kan <sup>R</sup>	This study
P <sub>trc</sub> -YFP	P <sub>trc</sub> promoter, YFP, <i>psbA2</i> integration site, Kan <sup>R</sup>	This study
P <sub>trc</sub> -YFP-E*	P <sub>trc</sub> promoter, YFP, riboswitch E*, <i>psbA2</i> integration site, Kan <sup>R</sup>	This study
pCA2.4-P <sub>trc</sub> -YFP	P <sub>trc</sub> promoter, YFP, pCA2.4 integration site, Kan <sup>R</sup>	This study
P <sub>psbA2</sub> -GAL	P <sub>psbA2</sub> promoter, $\beta$ -galactosidase, <i>psbA2</i> integration site, Kan <sup>R</sup>	This study
P <sub>trc</sub> -GAL	P <sub>trc</sub> promoter, $\beta$ -galactosidase, <i>psbA2</i> integration site, Kan <sup>R</sup>	This study
P <sub>trc</sub> -GAL-E*	P <sub>trc</sub> promoter, $\beta$ -galactosidase, riboswitch E*, <i>psbA2</i> integration site, Kan <sup>R</sup>	This study

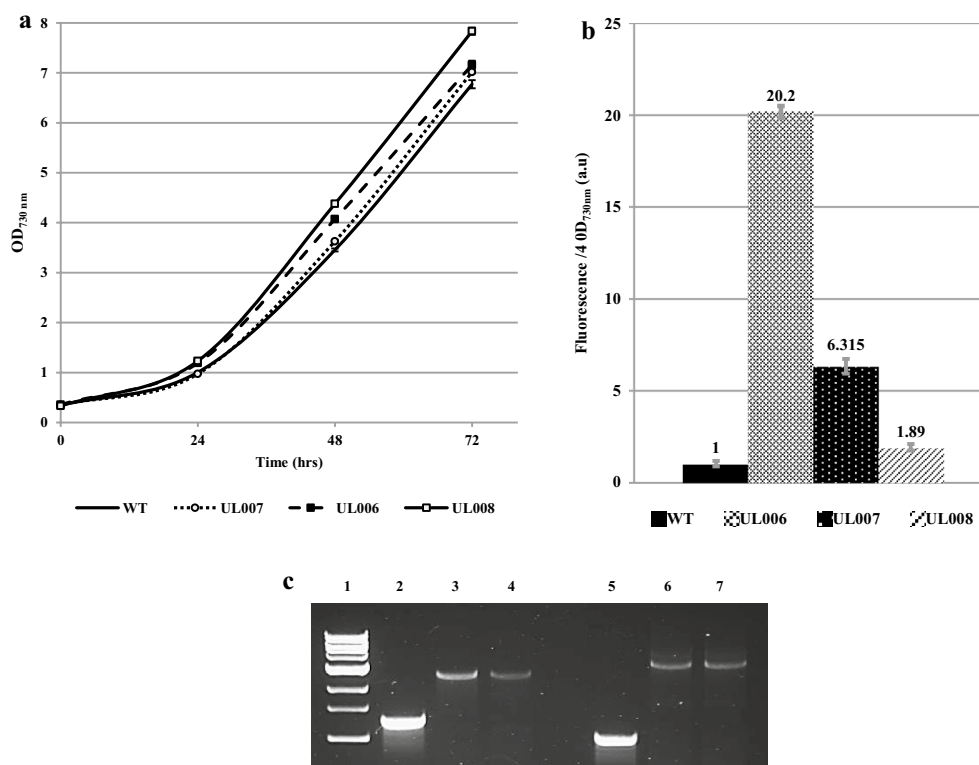


combinations were trialled; Ptc, Ptc-E\* and *Ppsba2*. Ptc is a strong promoter that is IPTG inducible derived from the *Escherichia coli* trp and lacUV5 promoters [21]. In this case, the Ptc promoter was modified to remove its repressor thereby allowing constitutive expression of the report gene. Ptc-E\* is a Ptc promoter linked to a riboswitch (E\*) that has been shown to allow inducible expression in *Synechococcus elongatus* PCC7942 through the use of the inducer compound theophylline [18]. *PpsbA2* is a native *Synechocystis* PCC6803 light inducible promoter regularly utilised for expression of genes in *Synechocystis* PCC 6803 [22]. As can be seen (Fig. 2b), the highest level of fluorescence was observed with the Ptc promoter. As noted earlier, the introduction of a riboswitch, while allowing inducible control of the Ptc promoter in *Synechocystis* PCC 6803, even when induced, reduced the maximum fluorescence level of the promoter significantly from a potential 20.2 to 6.32 au (Fig. 2b). In addition, it was found that the regularly utilised *Ppsba2* promoter expressed YFP at a significantly lower level

than the constitutive Ptc promoter: 1.89 au compared to 20.2 au. Hence, a Ptc-YFP combination was determined as the optimal construct for integration within the chromosomal locus *psba2* and the predicted pCA2.4 plasmid neutral site (Fig. 2b).

**Optimisation of the transformation and segregation conditions**

Introduction of foreign DNA into *Synechocystis* PCC6803 can be carried out via natural transformation [23]. As each cell contains multiple chromosome copies and non-native transformed plasmids can be lost during cell division, integration of foreign DNA into the chromosome is the preferred method. However, as the normal doubling time of the strain can be up to 20 h and each cell contains multiple copies of the chromosome, obtaining a fully segregated strain with a copy of the introduced DNA in each chromosome can take several weeks. In addition, several sub-cultures of the transformed colonies with increasing concentration of a selective pressure, usually a linked



**Fig. 2** Relative fluorescence levels of screened promoters and stability of modified pCA2.4 strains. **a** Optical density measurements at OD<sub>730nm</sub> of growth of different YFP-expressing strains over 72 h. Chromosomally integrated (*psbA2* locus) YFP strains UL006 (P<sub>trc</sub>-YFP), UL007 (P<sub>trc</sub>-YFP-E\*) and UL008 (P<sub>psbA2</sub>-YFP) grow similarly to WT. **b** Measurement of UL006, UL007 and UL008 YFP fluorescence levels after 72 h. The highest YFP fluorescence level detected was in UL006. Note 2 mM of the inducer theophylline was utilised to induce expression of the P<sub>trc</sub> riboswitch in UL007. **c** UL018 and UL006 strain stability after 80 days culturing. Lane 1 marker, lane 2 wild-type, lane 3 UL006 minus kanamycin, lane 4 UL006 plus kanamycin, lane 5 wild-type, lane 6 UL018 minus kanamycin, lane 7 UL018 plus kanamycin. The lower band indicates the presence of WT; higher band indicates construct integration. No evidence of the WT band in transconjugant strains was observed

antibiotic resistance gene, is necessary for complete segregation. However, if a glucose tolerant sub-strain of *Synechocystis* PCC6803 is utilised [24], the incorporation of glucose into the nutrient media can increase the growth rate of the strain and minimise the time between sub-cultures. In this analysis, the generation of a fully segregated strain containing the P<sub>trc</sub>-YFP cassette within the *psbA2* neutral site took 28 days under autotrophic conditions and 16 under mixotrophic conditions. In each case, transformed colonies were initially cultured on 5 µg ml<sup>-1</sup> kanamycin and sub-cultured to 25 µg ml<sup>-1</sup> and then 50 µg ml<sup>-1</sup> before full segregation occurred. Without glucose, a second sub-culture on 50 µg ml<sup>-1</sup> kanamycin was required to generate fully segregated strains.

Conversely, as the native plasmid pCA2.4 has an increased copy number under mixotrophic conditions [16], the incorporation of glucose prevented the generation of fully segregated strains. Figure 1e shows the screening of such transformant colonies. Note that in any conditions that incorporated glucose, no higher PCR

band indicating the presence of P<sub>trc</sub>-YFP construct integration in the pCA2.4 plasmid predicted neutral site can be observed. In addition, likely due to the higher copy number of the plasmid relative to the chromosome, additional sub-cultures and a higher antibiotic concentration were necessary for complete segregation of pCA2.4 transformants. Several pCA2.4 transformation attempts were carried out to optimise the transformation process. It was found that multiple further sub-cultures (up to 4) at high antibiotic concentration (50–100 µg ml<sup>-1</sup> kanamycin) in liquid media were necessary to achieve full segregation of pCA2.4 transformants (Fig. 1e).

As little is known about pCA2.4 and its function within the cell, the neutral site utilised within the plasmid may influence the ability to obtain a fully segregated stable strain [25]. Recently, Ng et al. [25] attempted to integrate a YFP cassette into 6 different predicted neutral sites within three different native plasmids of *Synechocystis* PCC6803. For 5 of these predicted neutral sites, detectable integration was not observed. For the 6th,



within native plasmid pCC5.2, a semi-segregated strain was obtained. No integration within the native plasmid pCA2.4 was detected [25]. In our study, full segregation of modified pCA2.4 plasmids could be achieved. This could be due either to the additional sub-cultures being carried out at high antibiotic concentrations in liquid media or the utilisation of a different predicted neutral site to those attempted by Ng et al. [25]. Thus, the choice of the neutral site may be quite important. In the case of Ng et al. [25], the unsuccessful neutral sites chosen were either within a predicted promoter region (1747–1819 bp, GenBank ID: L13739.1) or close to a predicted promoter region (390–523 bp, GenBank ID: L13739.1) [15, 25]. This may have contributed to the inability to integrate into the plasmid and generate stable transformants.

#### Expression levels achievable from pCA2.4 compared to chromosomal insertion

As can be seen (Fig. 2c), integration of the P<sub>trc</sub>-YFP cassette within pCA2.4 and the chromosomal site *psbA2* was stable; no reversion to wild-type was observed over 80 days, during which 12 sub-cultures with no antibiotic selection were carried out. No inhibitory effects on growth were observed (Fig. 3b) with both UL006 (chromosomal P<sub>trc</sub>-YFP) and UL018 (pCA2.4 P<sub>trc</sub>-YFP) growing similarly to wild-type. In addition, it was found that modification of the plasmid did not significantly affect the pCA2.4 per chromosome copy number. Significantly, YFP fluorescence from pCA2.4 was far higher than the chromosomal insertion, ranging from approximately 20 fold (day 6, without kanamycin) to 100 fold (day 14, with kanamycin added) higher YFP fluorescence detected from pCA2.4 over 14 days (Fig. 3a). For representative images of the difference in expression between chromosomal and plasmid integration site, see Fig. 4. It is likely that the difference in YFP fluorescence over the 14-day period was due to the changing copy number of pCA2.4 relative to the chromosome number during the cell cycle [16]. Overall, this analysis demonstrated that it is possible to achieve higher levels of gene expression from integration of a cassette into pCA2.4 relative to the chromosome. In addition, this analysis was carried out several times, both with and without selective pressure and yet no loss of YFP fluorescence was detected in the time period analysed indicating that integration was highly stable.

To examine the stability of integration, the growth and YFP fluorescence levels of several sequential sub-cultures of UL006 and UL018 without selective pressure were measured (Fig. 5). As can be seen, similar growth and YFP fluorescence levels were observed for each

sub-culture. Therefore, it can be concluded that integration within the native plasmid may be at least as stable as integration within a chromosomal locus even though the expression level from pCA2.4 far exceeds that of a chromosomal locus.

Interestingly, in the absence of selective pressure, the overall detected YFP fluorescence level from both UL006 and UL018 was marginally reduced (Fig. 3a) yet no reversion to wild-type was detected. As all samples were normalised to the same optical density before measurement, this indicated that it is possible that the inclusion of a selective pressure may increase both the chromosomal and plasmid copy number leading to overall higher YFP fluorescence levels. Without a selective pressure, YFP fluorescence from pCA2.4 was still higher than the chromosomal insertion, ranging from approximately 17 fold (day 4) to 67 fold (day 9) (Fig. 3a). In some cases, it may in fact be more favourable to culture modified pCA2.4 strains without a selective pressure to reduce the expression level, especially if the gene being expressed causes cellular stress at very high expression levels.

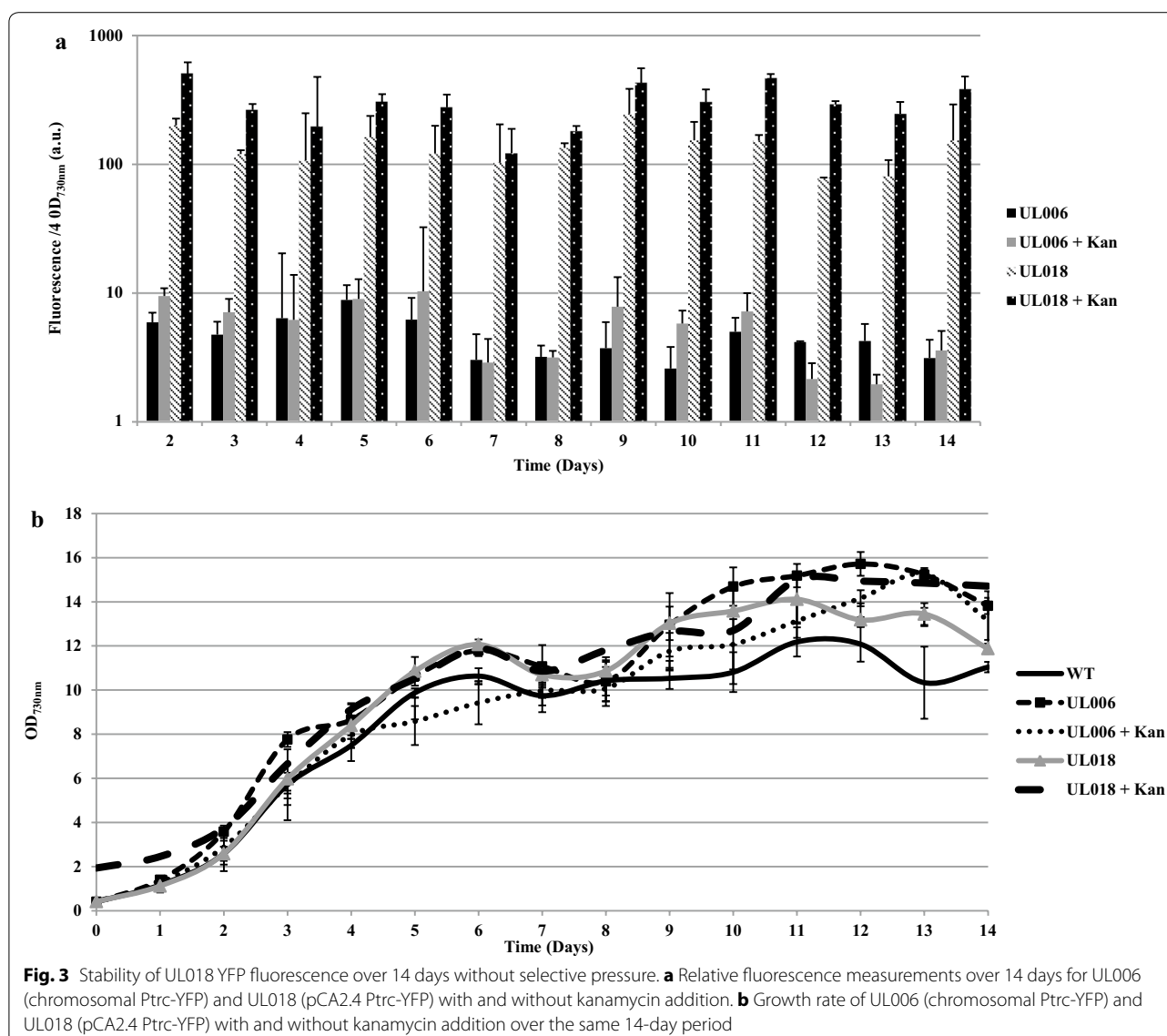
#### Conclusions

pCA2.4 is a stably maintained native plasmid within *Synechocystis* PCC6803. It is amenable to genetic modification and can be utilised to express selected genes at levels in excess of those achievable when compared with a chromosomal insertion. With the utilisation of an appropriate promoter, insertion into pCA2.4 may have several benefits; reduction of the time required for multi-copy modification; potentially minimisation of the requirement for antibiotics as only one modification is required to obtain multiple copies; potentially increasing stability compared to multiple chromosomal insertions as the native plasmid appears to be stably maintained. Finally, utilisation of the pCA2.4 neutral site may improve the maximum expression levels achieved with inducible promoter systems in *Synechocystis* PCC 6803 and maintain the advantages of an inducible system (Additional file 1: Figure S1).

#### Methods

##### Bacterial strains, DNA elements and media

The bacterial strains, plasmids and DNA elements utilised as part of this study are listed in Table 1. *Synechocystis* PCC6803 (glucose tolerant, obtained from K. Hellingwerf, UvA, Amsterdam) cells were routinely maintained at 30 °C on BG-11 media supplemented with 10 mM TES-NaOH (pH 8.2), 20 mM glucose and 0.3 % (w/v) sodium thiosulfate. All routine plasmid construction and cloning was performed in *E. coli* using Luria-Bertani (LB) broth. All media were supplemented with appropriate antimicrobial agents as required: ampicillin,

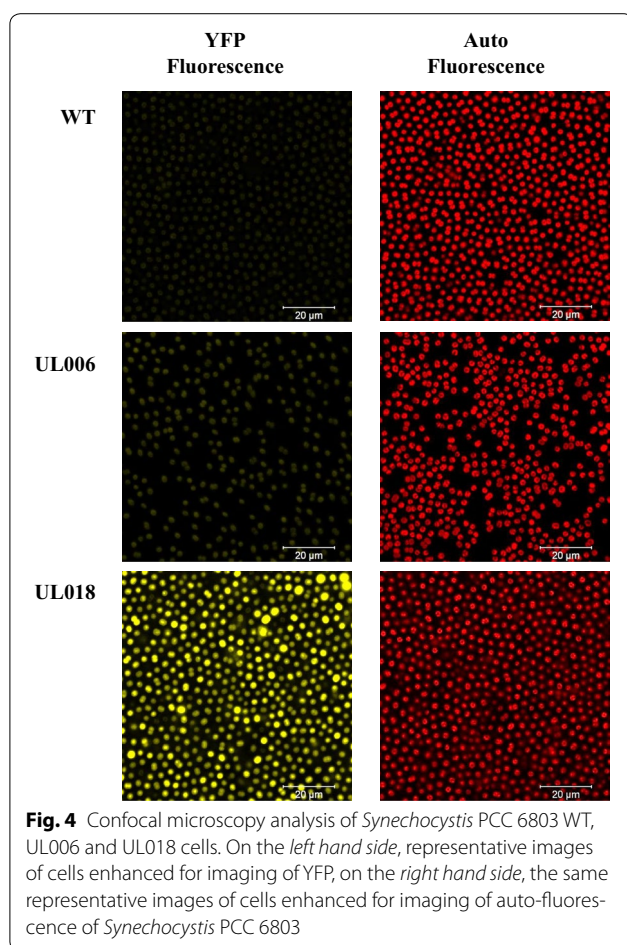


100  $\mu\text{g ml}^{-1}$ ; kanamycin, 5–100  $\mu\text{g ml}^{-1}$ , streptomycin, 100  $\mu\text{g ml}^{-1}$ . All strains were stored at  $-80^\circ\text{C}$  in either Luria–Bertani (LB) broth containing 50 % glycerol (*E. coli*) or 50 % BG-11 media containing 5 % (v/v) methanol (*Synechocystis* PCC6803).

#### Plasmid construction

All plasmids used in this study are listed in Table 1. All constructs were generated by utilising the In-Fusion method (Clontech, Mountain View, CA, USA) and verified by sequencing. Briefly, the In-Fusion enzyme premix was utilised to fuse PCR-generated sequences and linearised plasmid via homologous recombination at flanking 15 bp overlap sequences. The 15 bp overlap on both the PCR-generated sequences and

linearised plasmids were generated via the utilisation of customised primers. The pUC18 plasmid was utilised as the backbone for all generated constructs. The YFP and  $\beta$ -galactosidase source plasmids are listed in Table 1. The pTRC-E\* promoter sequence was obtained [18] and synthesised by Eurofins mwg/operon (85560 Ebersberg, Germany). pCA2.4 and *psbA2* homologous recombination sites were obtained by PCR with appropriate primers from AA314 [24]. The P<sub>trc</sub> constructs were generated by inverse PCR of a P<sub>trc</sub>-E\* construct to delete the E\* riboswitch. The *PpsbA2* promoter was obtained by PCR with appropriate primers from AA314. The kanamycin resistance gene was obtained from an integrative conjugative element termed ICE R391 [26].



### Transformation to *Synechocystis* PCC6803

*Synechocystis* PCC6803 cells were routinely cultured at 30 °C, 150 rpm, 2 % CO<sub>2</sub> (Multitron Pro, Infors HT) in BG-11 liquid medium supplemented with 1 g L<sup>-1</sup> HEPES–NaOH (pH 8.9) under high intensity white-light illumination (~80–100 μE m<sup>-2</sup> s<sup>-1</sup>). Growth was monitored at OD<sub>730nm</sub>. Cells were grown to mid-exponential phase (OD<sub>730nm</sub> of 0.4–1) and natural transformation performed as previously described with some modifications [23]. Briefly, 20 ml of cells were pelleted, washed in BG-11 media, pelleted and re-suspended in 1 ml BG-11 media to an approximate concentration of OD<sub>730nm</sub> equals 10. 100 μl of cells was then mixed with 20 μl of plasmid DNA (concentration range of 100–300 ng μl<sup>-1</sup>) in a 1.5-ml tube. Transformations were left at 30 °C for 16 h under medium intensity white-light illumination (~20–40 μE m<sup>-2</sup> s<sup>-1</sup>). Transformants were then plated directly onto BG-11 media containing kanamycin, 5 μg ml<sup>-1</sup>, ±20 mM glucose and grown for 5–10 days under medium light at 30 °C. Approximately, 40 of the resultant colonies were then streaked onto fresh BG-11 media containing increasing concentrations of antibiotic

as a selective pressure to promote construct integration and full segregation. For pCA2.4 transformations, further sub-cultures to liquid BG-11 media were required to achieve full segregation. Screening for full segregation was performed by PCR. Fully segregated colonies were verified by PCR and subsequent DNA sequencing of the PCR product. The plasmid pCA2.4 modified strain (UL018) was also verified to have no chromosomal integration at the *psbA2* locus via PCR and subsequent DNA sequencing.

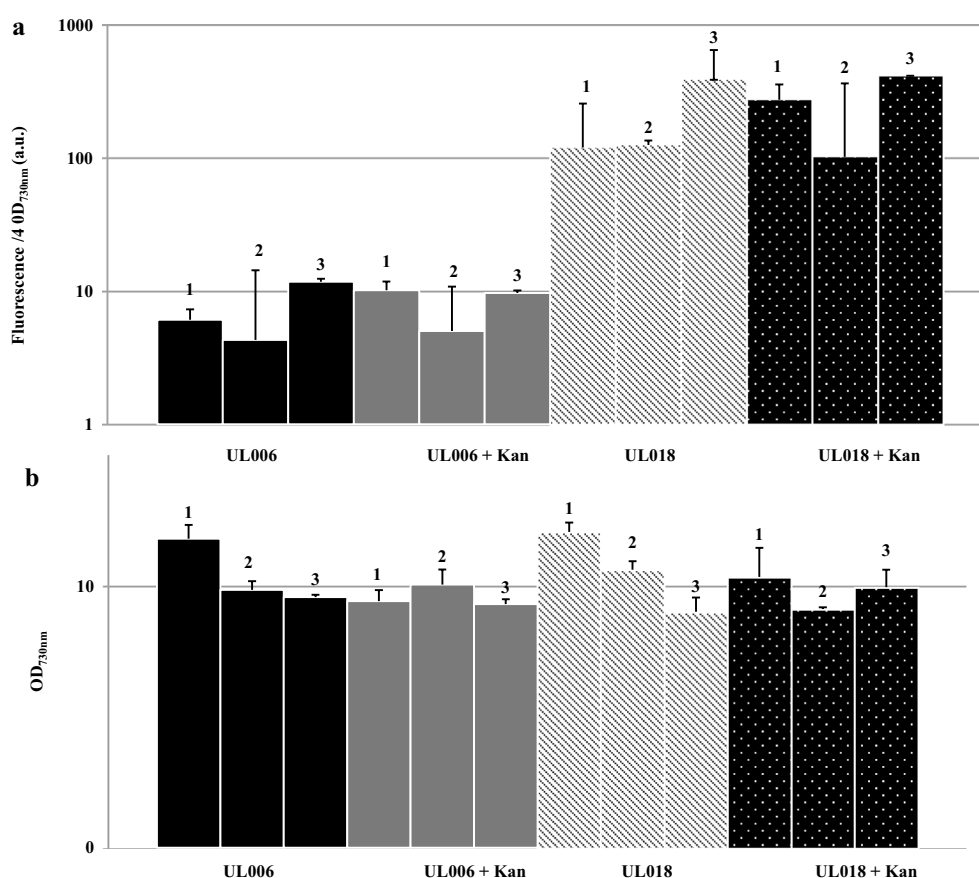
### Measurement of YFP fluorescence in *Synechocystis* PCC6803 transconjugants

All cultures were inoculated to OD<sub>730nm</sub> = 0.4 and cultured with high light intensity and 2 % CO<sub>2</sub> for 48 h. The culture OD<sub>730nm</sub> was then measured and the equivalent of 1 ml samples normalised to OD<sub>730nm</sub> = 4 were pelleted and treated with 300 μL of cell lysis buffer (Bugbuster, Cat # 70584-3, Merck Millipore) and left rocking gently for 30 min at room temperature. After lysis, samples were centrifuged at 13,000 rpm for 30 min to remove cell debris. Subsequently, samples of the supernatant were examined for fluorescence (513 nm excitation/532 nm emission) with a fluorescence spectrophotometer (Carey Eclipse, Agilent Technologies). Sample fluorescence was normalised against appropriate wild-type control samples. All reported fluorescence measurements are relative to wild-type. All data presented are the average of triplicate samples; all experiments were repeated a minimum of three separate times. For examination of strain stability without antibiotics, seed cultures were centrifuged at 4000 rpm for 15 min, washed in BG-11 media twice and re-suspended in BG-11 media ± kanamycin 50 μg ml<sup>-1</sup>. In addition, the average per chromosome copy number of pCA2.4 was confirmed to be similar to wild-type in UL006 and UL018 ± kanamycin according to the methods of Lee et al. 2006 [27].

### Confocal laser scanning microscopy of *Synechocystis* PCC6803

Cell samples were visualised using a Meta 710 laser scanning confocal microscope (Carl Zeiss, Germany) with a 63× oil immersion objective. The argon laser line (514 nm) and the helium–neon laser line (633 nm) were used for the excitation of enhanced YFP and auto-fluorescence respectively. The active spectral detection windows were configured to be 518–621 and 637–757 nm for enhanced YFP and auto-fluorescence respectively. Image processing was performed using the ZEN 2008 software package (Carl Zeiss, Germany). For each experiment, imaging parameters (laser excitation, photo multiplier tube gain, pinhole, electronic zoom size, etc.) were maintained at the same level to allow correct comparison. All





**Fig. 5** Growth rate and YFP fluorescence levels from 3 sub-cultures of UL006 and UL018. **a** Relative fluorescence measurements of three sub-cultures of UL006 and UL018 taken after 6 days growth without selective pressure (no kanamycin). **b** Optical density measurements at OD<sub>730nm</sub> of three sub-cultures of UL006 and UL018 taken at the same time point

cultures were inoculated to OD<sub>730nm</sub> = 0.4 and cultured with high light intensity and 2 % CO<sub>2</sub> for 72 h. The culture OD<sub>730nm</sub> was then measured and all samples were normalised to the same OD<sub>730nm</sub> and visualised.

## Additional file

**Additional file 1: Figure S1.** Plasmid map for pCA2.4 showing site of P<sub>trc</sub>-YFP construct integration into UL018. Two putative ORFs [*repA* and ORF2] are present within the native plasmid pCA2.4. P = predicted promoter, Term = predicted transcription terminator. The pCA2.4 neutral site utilised was from position 227 bp to 465 bp [GenBank ID: L13739.1].

## Abbreviations

AU: arbitrary unit; BG-11: bluegreen medium; DNA: deoxyribonucleic acid; GAL: β-galactosidase; HEPES: N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); OD: optical density; ORFs: open reading frames; PCR: polymerase chain reaction; WT: wild-type; TES: N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; YFP: yellow fluorescent protein.

## Authors' contributions

CS, JTP and PA participated in the design, coordination, drafting and revision of this manuscript. PA constructed all strains described in the manuscript,

performed laboratory work and analysed the resulting data. DC performed laboratory work including the measurement of YFP fluorescence in *Synechocystis* PCC6803 transconjugants. PA, DC, CS and JTP read and approved the final manuscript.

## Author details

<sup>1</sup> Molecular and Structural Biochemistry Laboratory, Department of Chemical and Environmental Sciences; Materials and Surface Science Institute, University of Limerick, Limerick, Ireland. <sup>2</sup> Department of Manufacturing and Operations Engineering, Faculty of Science and Engineering, University of Limerick, Limerick, Ireland.

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## Competing interests

The authors declare that they have no competing interests.

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## References

- Abas N, Kalair A, Khan N. Review of fossil fuels and future energy technologies. *Futures*. 2015;69:31–49. doi:10.1016/j.futures.2015.03.003.
- Sarsekeyeva F, Zayadan BK, Ussebaeva A, Bedbenov VS, Sinetova MA, Los DA. Cyanofuels: biofuels from cyanobacteria. Reality and perspectives. *Photosynth Res*. 2015;125(1–2):329–40. doi:10.1007/s11120-015-0103-3.
- Dexter J, Armshaw P, Sheahan C, Pembroke JT. The state of autotrophic ethanol production in Cyanobacteria. *J Appl Microbiol*. 2015;119(1):11–24. doi:10.1111/jam.12821.
- Angermayr SA, Rovira AG, Hellingwerf KJ. Metabolic engineering of cyanobacteria for the synthesis of commodity products. *Trends Biotechnol*. 2015;33(6):352–61. doi:10.1016/j.tibtech.2015.03.009.
- Watabe K, Mimuro M, Tsuchiya T. Development of a high-frequency in vivo transposon mutagenesis system for *Synechocystis* sp PCC 6803 and *Synechococcus elongatus* PCC 7942. *Plant Cell Physiol*. 2014;55(11):2017–26. doi:10.1093/pcp/pcu128.
- Guerrero F, Carbonell V, Cossu M, Correddu D, Jones PR. Ethylene synthesis and regulated expression of recombinant protein in *Synechocystis* sp PCC 6803. *PLoS One*. 2012;7(11):11. doi:10.1371/journal.pone.0050470.
- Camsund D, Heidorn T, Lindblad P. Design and analysis of LacI-repressed promoters and DNA-looping in a cyanobacterium. *J Biol Eng*. 2014;8:23. doi:10.1186/1754-1611-8-4.
- Griese M, Lange C, Soppa J. Ploidy in cyanobacteria. *FEMS Microbiol Lett*. 2011;323(2):124–31. doi:10.1111/j.1574-6968.2011.02368.x.
- Gudmundsson S, Nogales J. Cyanobacteria as photosynthetic biocatalysts: a systems biology perspective. *Mol Biosyst*. 2015;11(1):60–70. doi:10.1039/c4mb000335g.
- Gao ZX, Zhao H, Li ZM, Tan XM, Lu XF. Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria. *Energy Environ Sci*. 2012;5(12):9857–65. doi:10.1039/c2ee22675h.
- Kamennaya NA, Ahn S, Park H, Bartal R, Sasaki KA, Holman HY, et al. Installing extra bicarbonate transporters in the cyanobacterium *Synechocystis* sp PCC6803 enhances biomass production. *Metab Eng*. 2015;29:76–85. doi:10.1016/j.jymben.2015.03.002.
- Angermayr SA, van der Woude AD, Correddu D, Vreugdenhil A, Verrone V, Hellingwerf KJ. Exploring metabolic engineering design principles for the photosynthetic production of lactic acid by *Synechocystis* sp PCC6803. *Biotechnol Biofuels*. 2014;7:15. doi:10.1186/1754-6834-7-99.
- Jelenic S. Controversy associated with the common component of most transgenic plants—Kanamycin resistance marker gene. *Food Technol Biotech*. 2003;41(2):183–90.
- Jones PR. Genetic instability in cyanobacteria—an elephant in the room? *Front Bioeng Biotechnol*. 2014;2:12. doi:10.3389/fbioe.2014.00012.
- Yang XY, McFadden BA. A Small Plasmid, pCA2.4, from the cyanobacterium *Synechocystis* sp. strain PCC 6803 encodes a rep protein and replicates by a rolling circle mechanism. *J Bacteriol*. 1993;175(13):3981–91.
- Berla BM, Pakrasi HB. Upregulation of plasmid genes during stationary phase in *Synechocystis* sp strain PCC 6803, a cyanobacterium. *Appl Environ Microb*. 2012;78(15):5448–51. doi:10.1128/aem.01174-12.
- Ma AT, Schmidt CM, Golden JW. Regulation of gene expression in diverse cyanobacterial species by using theophylline-responsive riboswitches. *Appl Environ Microb*. 2014;80(21):6704–13. doi:10.1128/aem.01697-14.
- Nakahira Y, Ogawa A, Asano H, Oyama T, Tozawa Y. Theophylline-dependent riboswitch as a novel genetic tool for strict regulation of protein expression in cyanobacterium *Synechococcus elongatus* PCC 7942. *Plant Cell Physiol*. 2013;54(10):1724–35. doi:10.1093/pcp/pct115.
- Gao H, Tang Q, Xij X-D. Construction of copper-induced gene expression platform in *Synechocystis* sp. PCC6803. *Acta Hydrobiol Sin*. 2007;31(2):240–4.
- Fang F, Barnum SR. Expression of the heat shock gene hsp16.6 and promoter analysis in the cyanobacterium, *Synechocystis* sp PCC 6803. *Curr Microbiol*. 2004;49(3):192–8. doi:10.1007/s00284-004-4340-5.
- Brosius J, Erfle M, Storella J. Spacing of the –10 and –35 regions in the Tac promoter—effect on its in vivo activity. *J Biol Chem*. 1985;260(6):3539–41.
- Formighieri C, Melis A. Regulation of beta-phellandrene synthase gene expression, recombinant protein accumulation, and monoterpene hydrocarbons production in *Synechocystis* transformants. *Planta*. 2014;240(2):309–24. doi:10.1007/s00425-014-2080-8.
- Zang XN, Liu B, Liu SM, Arunakumara K, Zhang XC. Optimum conditions for transformation of *Synechocystis* sp PCC 6803. *J Microbiol*. 2007;45(3):241–5.
- Savakis PE, Angermayr SA, Hellingwerf KJ. Synthesis of 2,3-butanediol by *Synechocystis* sp PCC6803 via heterologous expression of a catabolic pathway from lactic acid and enterobacteria. *Metab Eng*. 2013;20:121–30. doi:10.1016/j.jymben.2013.09.008.
- Ng AH, Berla BM, Pakrasi HB. Fine tuning of photoautotrophic protein production by combining promoters and neutral sites in *Synechocystis* 6803, a cyanobacterium. *Appl Environ Microb*. 2015;81:6857–63. doi:10.1128/AEM.01349-15.
- Armshaw P, Pembroke JT. Generation and analysis of an ICE R391 deletion library identifies genes involved in the element encoded UV-inducible cell-sensitising function. *FEMS Microbiol Lett*. 2013;342(1):45–53. doi:10.1111/1574-6968.12107.
- Lee CL, Ow DSW, Oh SKW. Quantitative real-time polymerase chain reaction for determination of plasmid copy number in bacteria. *J Microbiol Meth*. 2006;65(2):258–67.

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